0273-2289/94/4546—0429$03.00

Removal of Inhibition by Ammonium Ion in Nitrogenase-Dependent Hydrogen Evolution of a Marine Photosynthetic Bacterium, *Rhodopseudomonas* sp. Strain W-1S

KIYOHITO YAGI,*[,1] ISAMU MAEDA,[1] KENJI IDEHARA,[1] YOSHIHARU MIURA,[1] TOHRU AKANO,[2] KIYOMI FUKATU,[2] YOSHIAKI IKUTA,[3] AND HIROSHI K. NAKAMURA[3]

[1]*Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565 Japan;* [2]*The Kansai Electric Power Company, Inc., Amagasaki, Hyogo, Japan; and* [3]*Mitsubishi Heavy Industries, Ltd., Arai-cho, Hyogo, Japan*

ABSTRACT

The addition of NH_4Cl at concentrations of more than 1 m*M* completely inhibited nitrogenase-dependent hydrogen evolution using 1 m*M* succinate as a substrate in the marine photosynthetic bacterium *Rhodopseudomonas* sp. strain W-1S. However, cells could derepress nitrogenase within 6 h in the presence of NH_4Cl. The inhibition by 1 m*M* NH_4Cl was removed by increasing the concentration of the substrate for hydrogen evolution. The addition of L-methionine-D,L-sulfoximine (MSX), an inhibitor of glutamine synthetase (GS), also removed the inhibition by 1 m*M* NH_4Cl. These results indicated that under an argon gas phase, nitrogenase-dependent hydrogen evolution is inhibited by ATP-consuming GS activity in the presence of NH_4Cl. We isolated a mutant strain, CR-8, which is capable of active hydrogen evolution in the presence of 1 m*M* NH_4Cl.

Index Entries: Photosynthetic bacteria; hydrogen; nitrogenase; glutamine synthetase; *Rhodopseudomonas*.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Many photosynthetic bacteria are known to carry out nitrogenase-dependent hydrogen evolution in the absence of nitrogen gas or ammonium ion (*1,2*). The rate of hydrogen evolution by photosynthetic bacteria is much higher than that of photo- and dark-hydrogen evolution in photosynthetic microalgae, such as blue-green and green algae (*3*). However, unlike microalgae, the photosynthetic bacteria cannot utilize water as a substrate for hydrogen evolution. This is the reason why most research on biophotolysis has been concentrated only on microalgal hydrogen evolution. We have proposed a new biophotolysis system using a green alga and photosynthetic bacterium consisting of three main steps (*4*):

1. Photosynthetic starch accumulation in the green alga;
2. Dark algal fermentation to produce hydrogen and organic compounds, such as acetate and ethanol;
3. Further conversion of the organic compounds to hydrogen by the photosynthetic bacterium.

Since the evolution of hydrogen and oxygen is separated in this system, the inactivation of hydrogenase and nitrogenase are expected to be avoided. Moreover, the yield of hydrogen evolution can be improved by recovering hydrogen from the organic compounds by the combination with photosynthetic bacteria. We have isolated a unicellular marine green alga, *Chlamydomonas* sp. strain MGA161, exhibiting high activity in dark hydrogen evolution (*5*). The alga excretes acetic acid and ethanol during dark anaerobic fermetation. We also isolated a marine photosynthetic bacterium, *Rhodopseudomonas* sp. strain W-1S, which evolves hydrogen from acetate and ethanol by a nitrogenase-dependent reaction (*4*). Sustained hydrogen evolution was achieved with a high conversion yield of 8 mol H_2/mol of starch-glucose in an alternating light-dark cycle.

It is well known that the ammonium ion inhibits nitrogenase-catalyzed hydrogen evolution in many microorganisms. It has been reported that the synthesis of nitrogenase is repressed by the addition of NH_4Cl (*6*). The ammonium ion must be responsible for the long lag in the appearance, and for the low rate, of hydrogen evolution. In this study, we attempted to remove the inhibition by the ammonium ion in hydrogen evolution from *Rhodopseudomonas* sp. strain W-1S, for the further improvement of our biophotolysis system.

MATERIALS AND METHODS

Cultivation

Rhodopseudomonas sp. strain W-1S was cultivated aerobically at 30°C on a modified Okamoto medium (MOM, pH 8.0) containing 30 g of NaCl,

0.2 g of $CaCl_2 \cdot 2H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 20 mg of $FeSO_4 \cdot 7H_2O$, 40.8 mg of KH_2PO_4, 495 mg of K_2HPO_4, 0.1 mg of thiamine, 1 μg of cyanocobalamin, 263 mg of NH_4Cl, 2.86 mg of H_3BO_3, 1.81 mg of $MnCl_2 \cdot 4H_2O$, 0.22 mg of $ZnSO_4 \cdot 7H_2O$, 0.08 mg of $CuSO_4 \cdot 5H_2O$, 0.021 mg of Na_2MoO_4, 0.01 g of $CoCl_2 \cdot 6H_2O$, and 50 mg of EDTA disodium salt in 1 L, with the addition of 10 m*M* acetic acid and 10 m*M* ethanol, under illumination by incandescent lamps at 200 W/m².

Hydrogen Evolution

Cells were harvested at the mid-log phase of growth, and washed twice with the medium for hydrogen evolution (HEM), which was MOM with the addition of 50 μg/L biotin, 0.3 mg/L *p*-aminobenzoic acid and 0.5 mg/L nicotinic acid, and with the omission of NH_4Cl and cyanocobalamin. Cells were then resuspended with 10 mL HEM containing various substrates in test tubes (18 mm in diameter). The tubes were flushed with argon gas for 30 min and then incubated on a reciprocal shaker (100 rpm) at 30°C under illumination of 200 W/m². The amount of hydrogen evolved was measured by gas chromatography (model-164, Hitachi).

Assay of Nitrogenase

The nitrogenase activity was measured by the reduction of acetylene. The assay was done in L-shaped test tubes (15 mL) with 4 mL of cell suspension containing 50 m*M* sodium malate and 50 μg/mL chloramphenicol. The gas phase in the L-tube was argon containing 10% acetylene. Ethylene formed during 30 min of incubation in the light (200 W/m²) at 30°C was measured by gas chromatography (GC-12A, Shimazu).

Isolation of Mutant

Mutagenesis was carried out using ethyl methansulfate (EMS) as a mutagen by the method described by Shanmugam and Valentine (*7*). The wild-type of strain W-1S was cultivated and resuspended in medium for the mutation (MM), which was HEM containing 1% of sodium acetate, sodium malate, sodium succinate, and sodium pyruvate and 5 m*M* NH_4Cl. EMS was added at a final concentration of 1%. Cells were incubated in the light (200 W/m²) at 30°C for 90 min, and then in the dark at 48°C for 40 min. The cells were then resuspended in MM and incubated in the light at 30°C for 10 h. After resuspending the cells in HEM, the suspension was spread on plates containing various concentrations of L-methionine-D,L-sulfoximine (MSX) between 0.1 and 10 m*M*, 1% of sodium acetate, sodium malate, sodium succinate, and sodium pyruvate, 2 m*M* L-glutamine, 0.5 m*M* L-glutamic acid, 1 m*M* L-aspartic acid, and 1 m*M* L-methionine.

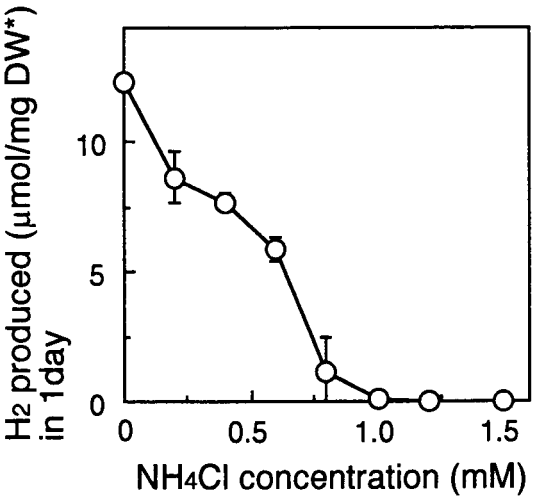

Fig. 1. Inhibition of hydrogen evolution by NH_4Cl in *Rhodopseudomonas* sp. strain W-1S. Various concentrations of NH_4Cl were added to HEM containing 1 m*M* sodium succinate as a substrate. The values are means ± SD of three experiments. *DW; dry weight.

RESULTS

The effect of the ammonium ion on hydrogen evolution in *Rhodopseudomonoas* sp. strain W-1S was examined using 1 m*M* sodium succinate as a substrate. As shown in Fig. 1, NH_4Cl inhibited hydrogen evolution in a concentration-dependent manner. The addition of NH_4Cl at concentrations of more than 1 m*M* completely inhibited hydrogen evolution. Figure 2 shows the changes of nitrogenase activity in HEM containing 1 m*M* sodium succinate in the presence or absence of 1 m*M* NH_4Cl. No nitrogenase activity was observed at the beginning of the incubation. In the absence of NH_4Cl, the activity was detected at 3 h and increased linearly with time. In the presence of NH_4Cl, the activity also appeared at 6 h and exceeded the activity in the absence of NH_4Cl at 12 h. The effect of NH_4Cl concentration on hydrogen evolution was examined using various concentrations of sodium succinate as the substrate. Figure 3 shows the results. Hydrogen evolution was not inhibited by 1 m*M* NH_4Cl in the presence of 5 m*M* succinate. In the case of the addition of 10 m*M* succinate, only slight inhibition was observed at 2 m*M* NH_4Cl. However, the activity was significantly inhibited by NH_4Cl at concentrations of more than 3 m*M*, even in the presence of a high concentration of succinate. We next examined the relationship between inhibition by NH_4Cl and glutamine synthetase (GS) activity. Cells were incubated in HEM containing 1 m*M* succinate for 24 h to induce nitrogenase. The effect of NH_4Cl on hydrogen evolution was then examined using MSX, an inhibitor of GS. As shown in Fig. 4, hydrogen evolution was completely inhibited at 1 m*M* NH_4Cl in

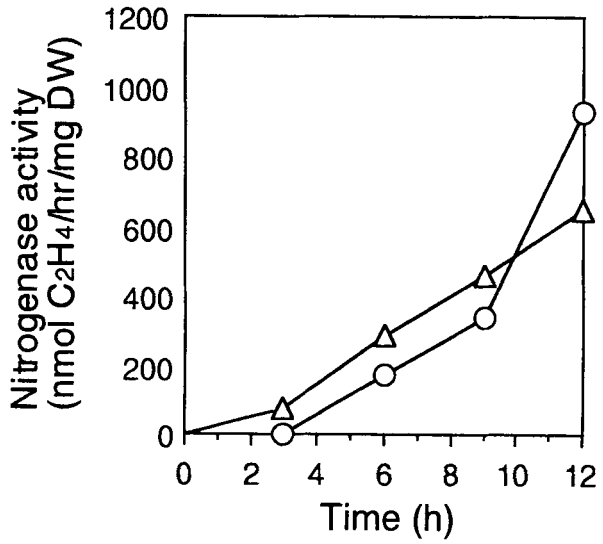

Fig. 2. Derepression of nitrogenase of *Rhodopseudomonas* sp. strain W-1S in the presence of NH_4Cl. Cells were incubated in the presence (○) or absence (△) of 1 m*M* NH_4Cl. At the times indicated, nitrogenase activity was measured by the acetylene reduction method described in Materials and Methods.

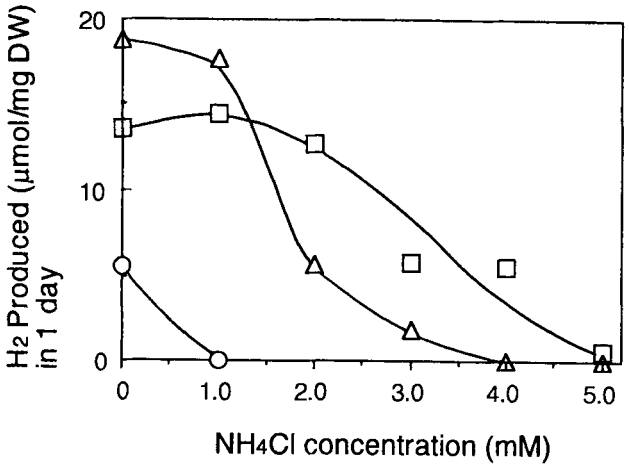

Fig. 3. Removal of NH_4Cl inhibition by increasing the substrate concentration. Cells were incubated in HEM containing 1 (○), 5 (△), and 10 (□) m*M* sodium succinate and various concentrations of NH_4Cl. The amount of hydrogen was measured at the times indicated.

the absence of MSX, while with the addition of 1 m*M* MSX, cells could evolve equivalent amounts of hydrogen at 1 m*M* NH_4Cl as that in the absence of NH_4Cl. These results showed that GS activity was responsible for the inhibition of hydrogen evolution by NH_4Cl. In order to isolate a mutant capable of hydrogen evolution in the presence of NH_4Cl, cells were treated with EMS as described in Materials and Methods. MSX-resistant

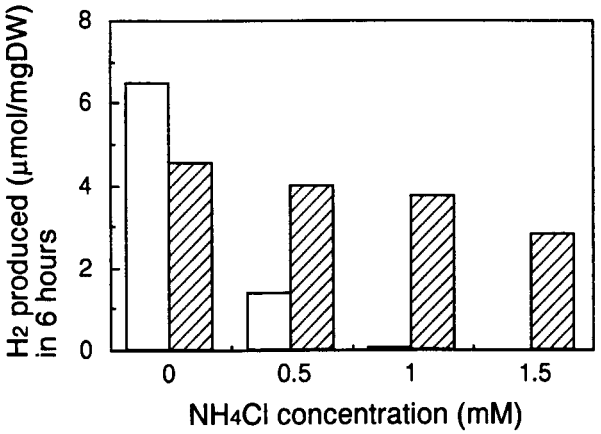

Fig. 4. Removal of NH_4Cl inhibition by MSX. Cells were incubated in HEM containing various concentrations of NH_4Cl in the presence (▨) or absence (□) of 1 m*M* MSX.

colonies were obtained on plates containing less than 4 m*M* of MSX. Ten colonies were selected and examined with respect to their hydrogen evolution activity in the presence of 1 m*M* NH_4Cl. Only one mutant, CR-8, had the activity. Figure 5 shows the effect of the ammonium ion concentration on hydrogen evolution of CR-8. When compared to the sensitivity in the wild type shown in Fig. 1, CR-8 showed significant resistance to NH_4Cl during hydrogen evolution with 1 m*M* succinate as the substrate.

DISCUSSION

The ammonium ion inhibited nitrogenase-dependent hydrogen evolution in the marine photosynthetic bacterium, *Rhodopseudomonas* sp. strain W-1S. This inhibition was not derived from the repression of nitrogenase synthesis by NH_4Cl, because there was high nitrogenase activity in the presence of 1 m*M* NH_4Cl. Besides nitrogenase activity, ATP and an electron donor are required for hydrogen evolution. It is possible that a deficiency in these factors in the presence of NH_4Cl caused the inhibition of hydrogen evolution. The concentration of sodium succinate, the substrate for hydrogen evolution, was raised to 5 and 10 m*M* to confirm this possibility. As expected, the inhibition caused by 1 m*M* NH_4Cl was completely removed by the addition of more than 5 m*M* succinate. However, as the concentration of NH_4Cl increased, the inhibition was observed even in the presence of 10 m*M* succinate. Therefore, it seems that nitrogenase competes with another ammonia-associated reaction for ATP or an electron donor. GS is the primary enzyme responsible for ammonia assimilation,

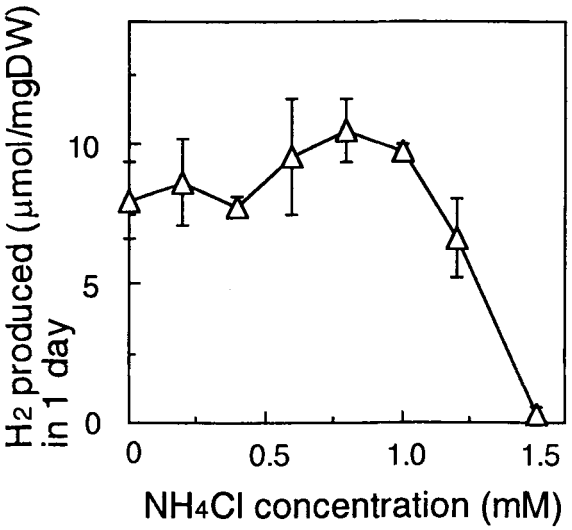

Fig. 5. Sensitivity to NH_4Cl of hydrogen evolution in mutant strain CR-8. Various concentrations of NH_4Cl were added to HEM containing 1 m*M* sodium succinate as a substrate. The values are means ± SD of three experiments.

and catalyzes the formation of L-glutamine from L-glutamic acid and ammonia using ATP. In the presence of NH_4Cl, GS would significantly affect nitrogenase-dependent hydrogen evolution by consuming ATP. To prove this hypothesis, the effect of NH_4Cl on hydrogen evolution was examined in the presence of MSX, an inhibitor of GS. Considering the toxic effect of MSX, nitrogenase was induced in advance and the amount of hydrogen evolved was measured after 6 h of incubation in the presence or absence of MSX. The addition of MSX completely removed the inhibition by 1 m*M* NH_4Cl. These results indicate that nitrogenase competes with GS for ATP in the presence of NH_4Cl; under an argon gas phase, nitrogenase-dependent hydrogen evolution is inhibited by ATP-consuming GS activity in the presence of NH_4Cl. Since MSX cannot be used in a practical application because of its toxic effect on cells and high cost, the isolation of a GS-less mutant is required for hydrogen evolution in the presence of NH_4Cl. Although nitrate is an excellent nitrogen source in microalgal growth, hydrogenase-dependent hydrogen evolution by microalgae was completely inhibited by the addition of nitrate, which acts as a terminal electron acceptor for respiration. Therefore, we want to use NH_4Cl as a nitrogen source for microalgal growth. In this study we isolated the mutant strain CR-8, which is less sensitive to NH_4Cl than the wild type. This kind of GS-less mutant will be very useful for improving our biophotolysis system, which consists of a microalga and a photosynthetic bacterium.

REFERENCES

1. Gest, H., Kamen, M. D., and Bregoff, H. M. (1950), *J. Biol. Chem.* **182,** 153.
2. Vignais, P. M., Colbeau, A., Willison, J. C., and Jouanneau (1985), *Adv. Microb. Physiol.* **26,** 155.
3. Mitsui, A., Philips, E. J., Kumazawa, S., Reddy, K. J., Ramachandran, S., Matsunaga, T., and Ikemoto, H. (1983), *Ann. N.Y. Acad. Sci.* **413,** 514.
4. Miura, Y., Saitoh, C., Matsuoka, S., and Miyamoto, K. (1992), *Biosci. Biotech. Biochem.* **56,** 751.
5. Miura, Y., Ohta, S., Mano, M., and Miyamoto, K. (1986), *Agric. Biol. Chem.* **50,** 2837.
6. Tubb, R. S. and Postgate, J. R. (1973), *J. Gen. Microbiol.* **79,** 103.
7. Shanmugam, K. T. and Valentine, R. C. (1980), *Methods Enzymol.* **69,** (Part C), 47.